

Studies on Antioxidant Induced
" Giantism and Cannibalism
of the Ciliated Protozoan,
Blepharisma americanum
(Suzuki, 1954)

By

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Table of Contents

	<u>Pgs.</u>
Acknowledgements	i.
Table of Contents	ii.
List of Figures and Photographic Plates.....	iii.
Abstract of the Dissertation	iv.
I. Introduction and Review of the Literature.	1.
II. Materials and Methods.....	6.
III. Observations and Results of Experimental Studies.....	17.
IV. Discussion and Conclusions.....	46.
V. Literature Cited.....	55.
VI. Appendices	
Appendix A: Formulary of Solutions.....	64.
Appendix B: Experimental Data.....	
Table II: Concentration Dependence of Tocopheryl--Induced Macrostomal Develop- ment.	65.
Table III: Influence of Temperature on Macrostomal Development	67.
Table IV: Influence of pH on Macrostomal Development.	68.

List of Figures and Photographic Plates

	<u>Pgs.</u>
Figure 1. Cannibalistic Feeding of <u>B. americanum</u>	19-20.
Figure 2. The Effects of Physical and Chemical Conditions on Macrostomal Development of <u>B. americanum</u>	32-33.
Figure 3. Frequency Distribution of Stages That Occur During Macrostomal Development.	34-35.
Plate I. Light Microscopic Study of Macrostomal Development.	22-23.
Plate II. Study of Macrostomal Development By Scanning Electron Microscopy.	26-27.
Plate III. Scanning Electron Microscopy (cont.).	28-29.
Plate IV. Transmission Electron Microscopy of Macronuclear and Nucleolar Changes During Macrostomal Development of <u>B. americanum</u>	39-40.
Plate V. A Survey of Macrostomal Ultrastructure of <u>B. americanum</u>	40-41.

STUDIES OF ANTIOXIDANT-INDUCED GIANTISM AND CANNIBALISM
OF THE CILIATED PROTOZOAN, Blepharisma americanum

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The present study was designed to find and develop a method for the induction of macrostomal development of the ciliated protozoan, Blepharisma americanum. This method was then used to study morphogenic and biochemical aspects of macrostomal production of this ciliate.

The antioxidant, d-alpha-tocopheryl, (vitamin E), was found to induce macrostomal development within 10-12 hours at 25°C when present at a final concentration of 10^{-4} M, at a pH of 5.0-5.5. Non-treated controls did not become giants. At lower concentrations of d-alpha-tocopheryl, or at lower temperatures, a lower percentage of the treated ciliates became macrostomes. At pH values less than 5.0 or greater than 5.5, fewer ciliates transformed into macrostomes.

The use of both light and scanning electron microscopy revealed that macrostomal development involves a series of increases in both the number and the size of the cilia comprising both the undulating membrane and the adoral zone of membranelles. The oral cavity also enlarges, forming a cytopharyngeal pouch for the capture and ingestion of prey. The prey-capture behavior is described. Macrostomal forms revert to the microstomatous morphology when giants are placed in fresh medium. This is accomplished by 2-3 successive cell divisions.

The production and the maintenance of the macrostomal form were found to be dependent on continuous protein synthesis. This was ascertained through the use of puromycin (100 mcg/ml/45min.) or cycloheximide (5.0 mcg/ml/45 min.) as inhibitors of protein synthesis. These drugs were used both during the transformation process and after its completion. Transmission electron microscopy revealed that the nucleoli of macrostomes increase both in number and in size as compared to controls. Also, nucleoli of giants demonstrate chains of ribosomes in close association with these nucleoli. Control organisms did not show such structures.

In addition to the above studies, the effectiveness of butylated hydroxytoluene (BHT), another antioxidant, as an inducer of macrostomal development was assessed. This substance induced macrostomal development (at 10^{-4} M) but the effect was transitory. Macrostomes reverted to the microrstomatous morphology within 4 hours.

A study of the use of d-alpha-tocopheryl as a protectant against the destructive effects of ultraviolet irradiation of B. americanum, showed that tocopheryl can be used to prevent cytolysis induced by ultraviolet radiation.

I.

INTRODUCTION AND LITERATURE REVIEW

Protozoa respond to changes in nutritional resources by a variety of methods, thereby increasing their chances for survival in a continually changing environment. One mechanism that enables ciliates to adapt to changes in their food supply, involves morphogenic transformations of the organism and a change in its feeding behavior. In particular, normally bacterivorous, small mouthed forms, known as microstomes, may transform into large-mouthed, carnivorous forms termed macrostomes. The latter form may also become cannibalistic. This transformation is exemplified by such ciliates as the hymenostome, Tetrahymena vorax (Buhse, 1966a,1966b,1967), & others listed in Table I.

The above noted changes in morphology and diet are preceded by a series of cellular transformations involving the oral apparatus of the ciliate. For Blepharisma americanum, there is a marked increase of the length of the ciliature comprising the undulating membrane situated on the right side of the buccal cavity and the adoral zone of membranelles, situated along the left margin of the buccal cavity. Also, the number of component cilia in each structure may increase in the giant form (Giese, 1938; Pierce et al., 1978; Repak, 1967). In most instances, the buccal cavity becomes greatly enlarged due to the fusion of the posterior portion of the cytopharynx with an accessory vesicle formed at the center of the organism. The cytopharyngeal pouch thus formed is

used to capture and ingest large prey (Buhse & Stamler, 1977 Buhse et al, 1978). This organelle remains intact and functions as long as the conditions for the maintenance of the macrostome are maintained.

A macrostome may continue to feed, grow and reproduce in that form as long as the environmental conditions are appropriate. However, if a population of macrostomes is placed in non-nutrient salt solutions or in bacterized medium, the giants return to the microstomatous form. The pink, heterotrich, Blepharisma americanum, reverts to the microstomatous form by a series of rapid cell divisions, resulting in 6-100 cells of normal proportions per giant (Giese, 1938 & these studies). In contrast, Tetrahymena vorax may resume the proportions of a microstome by partial resorption of the oral ciliature and a decrease in the volume of the buccal cavity, or the macrostome may divide to produce two microstomes (Buhse, 1966a). An inspection of published figures also shows that the cytopharyngeal pouch usually disappears during reversion (see references listed in Table I).

In addition to the morphological changes described above, macrostomal development also involves numerous changes of the biochemistry of the organisms undergoing the transformation. In Tetrahymena vorax V_2S and T. paravorax, synthesis of hydrolytic enzymes is increased (Metenier, 1977), new RNA and proteins required for the maintenance of the enlarged oral apparatus, are synthesized (Buhse & Cameron, 1968; Buhse and Nicollette, 1969, 1970), and the ciliate generally demon-

strates a decreased respiratory rate (Buhse & Hamburger, 1974; Buhse et al, 1974). These studies have been limited to macrostome forming species of the genus Tetrahymena due to the lack of a dependable technique for the induction of macrostomal development of ciliates such as Blepharisma americanum.

The research project forming the basis for this dissertation is directed toward the acquisition of additional information concerning the morphogenic and biochemical changes that occur when a tocopheryl (vitamin E) treated microstome transforms into a cannibal-giant macrostome.

In the first section of this project, the question of the morphogenic events required prior to the formation of the giant morphotype is considered. A combined light and scanning electron microscopy study is used to delineate the major stages involved in the alteration of the oral cavity and the associated ciliature. The pink heterotrich, Blepharisma americanum (Suzuki, 1954), was chosen for this work due to its known capacity for giantism (see Stolte, 1924; Dawson, 1929; Giese, 1938), the ease with which this organism can be cultivated in large numbers, and because of the current lack of knowledge concerning the details of the giant formation phenomenon of this ciliate.

In the second section of this research, data and observations are presented that serve to define optimal conditions for the induction of giantism of B. americanum by d-alpha-tocopheryl succinate. In particular, the parameters investigated included concentration of antioxidant, temperature

and pH.

Following the standardization of the induction technique, a survey of ultrastructural changes in macrostomes as contrasted with microstomes was conducted. Changes of macronuclear ultrastructure were of primary interest, since such changes may serve as indicators of metabolic changes related to the production of giants. An initial study of the effects of specific metabolic inhibitors (cycloheximide, puromycin and colchicine) was conducted as well.

For the final section of this project, the means by which giants revert to microstomatous proportions was investigated with light and scanning electron microscopy.

A model for the action(s) of tocopheryl in eucaryotic gene regulation is presented, based on an analysis of currently available information. Also, proposed avenues for future testing of this model are considered.

Gymnostomea		
<u>Dileptus anser</u>	Janovy, 1963	
Suctorea		
<u>Tokophrya infusionum</u>	Lilly, 1942; Rudzinska, 1953	
Vestibuliferea		
<u>Bresslaua vorax</u>	Stout, 1960	
<u>Woodruffia metabolica</u>	Johnson & Larson, 1938	
<u>Espejoia mucicola</u>	Fauré-Fremiet & Mugard, 1949	
Hypotrichea		
<u>Euplotes balteatus</u>	Tuffrau, 1959	
<u>Gastrostyla steini</u>	Weyer, 1930	
<u>Holostricha flavorolina</u>	Schaeffer, 1937	
<u>Oxytricha hymenostoma</u>	Dawson, 1919, 1952	
<u>Pleurotricha lanceolata</u>	Joukowsky, 1898	
<u>Stylonychia curvata</u>	Alonso & Perez-Silva, 1963; Giese & Alden, 1938	
Hymenostomea		
<u>Tetrahymena vorax</u>	Kidder <u>et al</u> , 1940; Claff, 1947	Buhse, 1966b, 1967; Buhse & Cameron
	Buhse, 1966a; Williams, 1961	1968; Buhse & Corliss, 1968, 1969
<u>T. paravorax</u>		Metenier, 1975, 1976, 1977, 1978; Meten-
		iér & Groliere, 1979
<u>T. patula</u>	Maupas, 1886; Miller & Stone, 1963	Stone, 1963
	Roux, 1901	
Heterotrichea		
<u>Blepharisma americanum</u>	Giese, 1938; Lennartz & Bovee, 1980	Pierce <u>et al</u> , 1978
<u>B. intermedium</u>	Dawson, 1929	Seshachar, 1964
<u>B. japonicum</u>	Nilsson, 1967	
<u>B. undulans</u>	Ibara, 1939; Padmavathi, 1959	Steinberg, 1959
<u>Stentor coeruleus</u>	Burchill, <u>et al</u> , 1981 unpublished.	
	von Gelei, 1925	

II

MATERIALS AND METHODS

I. Organisms Used In These Studies:

The ciliate chosen for these studies is the large, pink heterotrich, Blepharisma americanum (Suzuki, 1954). This organism was selected for study due to its large size (150 μ m x 50 μ m); the ease of cultivation in large numbers under well standardized conditions, and the known occurrence of cannibal-giantism in this species. Organisms used in these studies were originally isolated from dried Sphagnum moss of unknown geographic origin, by soaking the moss in Chalkley's solution (see Appendix A) for 5-7 days, then removing the excysted ciliates with a fine pipette. The specific identification of B. americanum was confirmed by consulting the taxonomic literature for the genus (Suzuki, 1954; Giese, 1973).

Organisms that had been identified were established in monoxenic bacterized cultures according to the following procedure:

A. Organisms were removed from the moss samples and placed in 2 ml. of Chalkley's solution in a watchglass.

B. Single cells were transferred via sterile micro-pipettes, through 5-10 washes of sterile Chalkley's solution to remove detritus and the majority of associated organisms (mainly bacteria).

C. Washed cells were transferred as a group to a mixture of antibiotics prepared in sterile Chalkley's solution. The antibiotics used were penicillin G (5,000 μ g/ml) and streptomycin sulfate (24 μ g/ml) as recommended by Marti, et al (1979). The ciliates were left in the antibiotic mix-

ture for 5 hours, then transferred to a medium consisting of Chalkley's solution, wheat grains (2-3/100ml) and the bacterium, Klebsiella aerogenes. The bacterial food source had been added 48 hours prior to the introduction of the B. americanum. The pH of the culture was adjusted to 6.0-6.5 with dilute NaOH as required.

D. Cultures of B. americanum and the bacterial food source, were both maintained at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ in a Fisher Low Temperature Incubator (Model # 146). The bacterial cultures were maintained in Petri dishes on 2% nutrient agar or in screw capped culture tubes (15 ml.) containing 2% proteose peptone (Difco).

E. Subcultures were started every two to three weeks to maintain B. americanum in the microstomatous form.

II. Preparation of Antioxidant Test Solutions.

A 10^{-4}M stock solution of d-alpha-tocopheryl succinate was prepared immediately before use by dissolving the crystalline vitamin (ICN Pharmaceuticals; Cleveland, Ohio, Lot # 1091) in 2-3 ml. of absolute ethanol and then adding Chalkley's solution to the required volume. Serial dilutions of the stock solution produced the following series of test-concentrations: 10^{-4}M , 10^{-5}M , 10^{-6}M & 10^{-7}M , with respect to d-alpha-tocopheryl. A second series of serial dilutions was prepared using the water-soluble vitamin E preparation, Aque-sol E (U.S. Vitamin Inc., Tuckahoe, New York. Form of dl-alpha-tocopheryl). The same range of concentrations was produced. The pH of these solutions was adjusted to 5.0-5.5 prior to use

by the addition of dilute NaOH.

In addition to vitamin E, the non-vitamin antioxidant BHT (2,6-ditert-butyl-p-cresol from Sigma Chem. Co., St. Louis, Mo.), was tested for its ability to induce giantism in B. americanum. This substance was tested over the same range of concentrations & pH as the tocopheryl mentioned above.

III. Test Procedure for Antioxidant Induction of Giantism.

For experimentation, B. americanum microstomes were selected from cultures (1.5 to 2 weeks after initiation) & washed at least 5 times in separate aliquots of sterile Chalkley's solution. This washing procedure removed the majority of adhering bacteria from the ciliates. Since random size variation has been thought previously to be a factor in giant formation (Pierce, et al, 1978), the washed cells were observed to confirm that the organisms to be treated were microstomes of approximately equal size (150 μm x 50 μm), and of normal morphology. A calibrated ocular micrometer was used for these measurements.

Organisms were transferred to watchglasses; each watchglass contained fifty ciliates in 2.0 ml. of the vitamin solutions to be tested. The cells were thoroughly mixed in the solution and the time recorded at the beginning of the tests. At hourly intervals, the ciliates were examined by light microscopy (brightfield, darkfield, phase contrast and Nomarski interference contrast methods). Photographic records of the transformation were made with an Olympus PM-

6, 35 mm camera or a Polaroid camera attached to the microscope in use (either a Zeiss RA or an Olympus BHC).

Besides the light microscopic observations made during the course of the transformation process, samples of treated cells were removed at hourly intervals (from a duplicate set of watchglasses) and stained for detailed observation of the oral ciliature by either the protargol impregnation method of Ng & Nelson, (1977) or by the CrO_3 -glutaraldehyde- OsO_4 method of Lennartz [see Appendix A].

Since the tocopheryl solutions contained small quantities of ethanol and succinate, a series of control experiments was devised to assess the extent of possible effects of these substances on treated ciliates. The controls consisted of 50 washed cells per watchglass of either (a) 1% ethanol, (b) 10^{-4}M succinate, or (c) A mixture of ethanol and succinate that was 1% with respect to ethanol and 10^{-4}M with respect to succinate. A fourth watchglass containing washed, untreated organisms was used to provide a basis for comparisons.

In general, all experimental tests were conducted for 10-12 hours at $25^\circ\text{C} \pm 0.5^\circ\text{C}$ in darkness. The pH was monitored hourly and adjusted as needed to 5.0-5.5. All experiments were conducted three times and the mean percentage of macrostomes formed per hour was determined in each case.

An additional study of the frequency distribution of five stages of macrostomal development was conducted, based on the data obtained from the above study.

IV. Effects of Temperature on the Rate of Macrostomal Development of *Blepharisma americanum*.

Assessment of the influence of temperature on the rate of macrostomal development induced by d-alpha-tocopheryl, was made according to the following procedure:

Organisms were isolated, and treated with vitamin E as described in Section III. This was done at 10°C, 20°C & 30°C with three trials at each temperature. The selected temperatures were maintained to within 0.5°C, by suitably adjusted incubators. Hourly samples were examined, and the mean percentage of macrostomes present per hour was determined.

Controls for this study consisted of one group of untreated organisms at each temperature.

V. The Effect of pH on Macrostomal Development.

Organisms were treated with 10^{-4} M d-alpha-tocopheryl succinate as detailed in Section III., except that triplicate samples of organisms (50 ciliates per sample) were exposed to the vitamin at different pH values: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5. The pH was adjusted with dilute NaOH or dilute HCl as required. The pH was monitored hourly with test strips. For each group of organisms at each pH, the mean percentage of macrostomes formed per hour was calculated.

VI. Effects of Starvation on Macrostomal Production.

Since the formation of a giant ciliate is known to require accelerated synthetic activities, the nutrition of the pre-transforming organism may play an important role in the response of that cell to an inducer. Also, nutrition would

be expected to affect the amount of precursor molecules available for biosynthetic reactions. To test this hypothesis, the following experiment was devised:

A. Microstomatous B. americanum were harvested, washed and divided into four groups, each containing 50 cells with the exception of the third group (150 cells).

The first group was fed continuously with bacteria before and during the experiment. The second group of ciliates was fed with bacteria and then exposed to 10^{-4} M d-alpha-tocopheryl-succinate without prior starvation. The third group was further subdivided into three subgroups of 50 cells per group. The first subgroup was starved for 6 hours; the second for 12 hours, and the third for 24 hours, prior to the start of the experiment. A fourth group of ciliates was starved similarly, but was then treated with 10^{-4} M d-alpha-tocopheryl succinate during the experiment.

B. Each group was examined hourly for changes toward macrostome formation and the percentage of transforming cells was determined. All groups were treated in darkness at 25°C, pH 5.0-5.5.

VII. Macronuclear Morphology of Microstomatous & Macrostomatous B. americanum.

Previous studies have noted that the number of nodes that comprise the macronuclear chain of B. americanum, is significantly greater for the macrostomatous form as compared to the microstomatous type (Hirshfield, et al, 1963). This observation was confirmed for organisms treated with d-alpha-toco-

pheryl-succinate by staining macrostomes and microstomes with the nucleic acid specific stain, methyl green-pyronin Y (Hudson, 1972).

VIII. Study by Scanning Electron Microscopy of Changes of the Oral Ciliature of Macrostomes as Compared to Microstomes.

In order to more accurately describe the changes that occur in the oral apparatus of a tocopheryl induced giant, the following scanning electron microscopic procedure was developed:

A. At intervals of 0,3,6,9 & 12 hours, following the addition of vitamin E, samples of ciliates were fixed in $\text{CrO}_3\text{-OsO}_4$ -glutaraldehyde for 30 minutes.

B. Fixed organisms were dehydrated in an ascending series of ethanol (40%, 80%, 95%, 100%a and 100%b) for 3 minutes at each stage.

C. Dehydrated organisms were attached to round coverslips with 0.25% polylysine, and then subjected to critical point drying in CO_2 using a Bomar SPS-1500 apparatus.

D. The coverslips were then affixed to cleaned specimen studs with silver conducting cement and sputter-coated with a Au/Pd mixture (200 Å thickness) in a Technics Hummer II, sputter coating apparatus.

E. Specimens were examined with a Philips 501 SEM, operating at 15 kV. Images were recorded on Polaroid P/N 55 sheet film.

IX. Study of Nucleolar Ultrastructure of Tocopheryl-treated Macrostromes and Untreated Microstromes.

The transmission electron microscope was used to study changes of nucleolar ultrastructure that occur as a result of macrostomal induction by d-alpha-tocopheryl-succinate. The following procedure was used for both untreated ciliates and ciliates exposed to the vitamin for 6 hours before preparation for TEM work:

A. Organisms were fixed in 2.5% glutaraldehyde buffered with 0.1M Na-cacodylate (pH=7.35), at 4°C for 1.5 hours. Cells were then washed twice with buffer.

B. Secondary fixation was done by exposure of the cells to 1% aqueous OsO₄ for 20 min. at 22°C.

C. Samples were dehydrated in an ascending series of ethanol (60%, 70%, 80%, 95%, 100% a, 100% b) for 10 minutes per change.

D. Cells were pelleted by centrifugation and embedded in Spurr's medium (Spurr, 1969) prior to sectioning. Silver-gray sections were cut using a Porter-Blum MT-2-B ultramicrotome equipped with a diamond knife.

E. Sections were picked up on 400 mesh, uncoated, copper specimen grids, and then double stained with lead-citrate (5 min.) and uranyl acetate (1 hr). Specimens were allowed to dry.

F. Sections were examined with a Philips 300 transmission electron microscope operating at 60 kV. Images were recorded on Kodak 4489 EM sheet film using photometrically determined exposure times.

X. Effects of Puromycin on Macronuclear & Nucleolar Organization of Tocopheryl Induced Macrostomes.

In order to investigate the nature of ribosome-like and polyribosome-like particles found in the nucleoplasm and in association with the nucleoli of macrostomes, the protein synthesis inhibitor, puromycin dihydrochloride (Sigma Chem. Co., St. Louis, Mo.), was used as follows:

A. A sample of tocopheryl-treated ciliates was examined to ensure that the organisms were macrostomatous, and then the sample was divided into two groups. One group was untreated and served as the control.

B. The second group of organisms was exposed to puromycin (100 mcg/ml) for 45 minutes at 22°C.

C. Both groups were prepared for transmission electron microscopy after the inhibitor was washed off of the treated cells. The procedure for TEM preparation was identical to that used in Section IX.

XI. Effects of Inhibitors of Protein Synthesis on the Maintenance of the Macrostomal Morphology of Blepharisma americanum.

In view of the observation that cycloheximide and puromycin both cause the collapse of the cytopharyngeal pouch of Tetrahymena vorax (Buhse, et al, 1978), the following experiment, using tocopheryl-induced macrostomatous B. americanum, was done to determine whether protein synthesis is required for the maintenance of the macrostomatous form of this cell:

A. Either puromycin (100 mcg/ml) or cycloheximide (5mcg/ml) [Both from Sigma Chem. Co.], was added to samples of washed

ciliates. The cells were observed by light microscopy for the disappearance of the cytopharyngeal pouch.

XII. Effects of Colchicine on the Maintenance of the Cytopharyngeal Pouch of Macrostomatous *B. americanum*.

In order to test the hypothesis that microtubules are involved in the maintenance of the cytopharyngeal pouch of tocopheryl-treated *B. americanum*; 10^{-6} M colchicine (final concentration) was added to a washed sample of ciliates (completed macrostomes). Observations were made with a Zeiss RA light microscope.

XIII. Initial Study of Tocopheryl-Mediated Protection of *B. americanum* Against Ultraviolet Radiation.

The sensitivity of *Blepharisma* to ultraviolet irradiation is well known (Giese, 1973). These ciliates possess a photosensitizing pigment, *blepharismine*, beneath their cell membranes. This pigment undergoes photooxidative reactions when it absorbs ultraviolet radiation. Such reactions are thought to generate free radicals that damage the cell membrane and lead to cytolysis (Giese, 1973).

The experiment described below is an initial investigation of the action of d-alpha-tocopheryl as a protective agent against ultraviolet radiation induced cell damage:

A. Triplicate sets of cells were washed, treated with 10^{-5} M d-alpha-tocopheryl-succinate and allowed to remain undisturbed for six hours in the dark. Two control groups without added vitamin E were prepared also.

B. The cells of both treated and non-treated groups were exposed to 254 nm ultraviolet radiation for 5-10 seconds

[Dosage= 36.67 joules/m²].

C. A light microscope was used for observation of each group.

III.

OBSERVATIONS AND RESULTS OF EXPERIMENTAL STUDIES

I. Study of Macrostomal Morphogenesis of Tocopheryl-Treated Blepharisma americanum by Light Microscopy.

The transformation of microstomatous B. americanum into cannibal-giants begins as early as 2.5 to 3.0 hours after the addition of 10^{-4} M d-alpha-tocopheryl-succinate (Temperature = 25°C , pH = 5.0-5.5). The first recognizable changes of transforming organisms are a pronounced shortening of the body from 150 μm to 100 μm and an increase of the width of the anterior end. At this time, some monsters of bizarre form occur (less than 1% of the treated organisms). The most unusual of these monsters is an L-shaped form that usually swims in tight circles. These L-shaped organisms survive for up to 48 hours, but do not form macrostomes. Such monsters, when isolated & fed the same bacteria as the controls, do not form clones and cannot feed cannivorously. These cells are apparently unable to feed for sustained periods of time and they do not divide. The cause(s) of such monsterism remain, as yet, unknown. These monsters have been seen previously under conditions where the inducer was not defined (Giese, 1938).

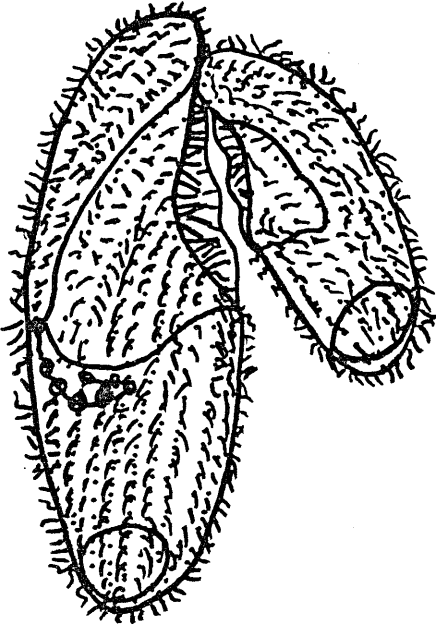
After four hours of exposure to the vitamin, the length of the oral apparatus increases until it becomes approximately 75 μm long. This is an increase of nearly 50% as compared to the controls. The increase in length occurs rapidly, within 3-5 minutes. The cilia of the adoral zone of membranelles and of the undulating membrane, become slightly shorter prior to the elongation of the oral apparatus. Also, the motility of the oral ciliature is considerably reduced for about 60 min. beginning at the time of elongation. The organisms do not

feed or move about extensively during that span of time.

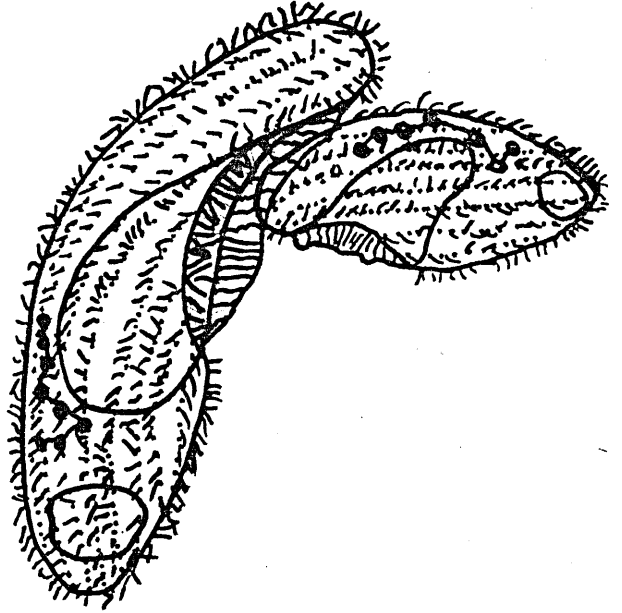
During the remainder of the 12 hour period, B. americanum continues to increase in overall length and body volume. Both the adoral zone of membranelles and the undulating membrane double in width, and after the hour of immobility, begin to beat with the strong, active metachronal pattern that characterizes the motility of the oral ciliature of this organism. From the 9th to the 12th hour, the cells are capable of cannibalistic feeding, as confirmed by the presence of large food vesicles containing partially digested B. americanum, and by the direct observation of prey capture by several cannibal-giants.

Cannibalism occurs when predator and prey organisms are oriented with their oral apparatuses closely apposed (Fig. 1a). At first, the organisms appear to be starting the agglutination phase of conjugation, but in several seconds it is clear that this is not the case. Instead, the somewhat smaller organism is forced toward the gaping cytostome of the predator by the active, repeated impact of the predator's anterior end with the antero-dorsal surface of the prey (Fig. 1b). This behavior, combined with the swimming of the predator and prey toward one another, makes capture of the prey possible. The prey is seized by the "grasping" action of the rim of the cytostome and the undulating membrane. Prey capture is always "anterior end first" (Fig. 1c). This prey capture sequence usually requires 3-5 minutes to complete, depending on the size of the prey. Following capture, the ingested prey is

Figure 1. Cannibalistic Feeding By Macrostomatous Blepharisma americanum. a. Two macrostomes become aligned with their oral apparatuses closely apposed. b. The larger of the two ciliates begins to push the smaller organism into the gaping cytostome by repeated contact of the predator's anterior end with the prey. c. The prey is captured. d. The ingested prey is enclosed within a food vesicle at the fundus of the cytopharyngeal pouch.



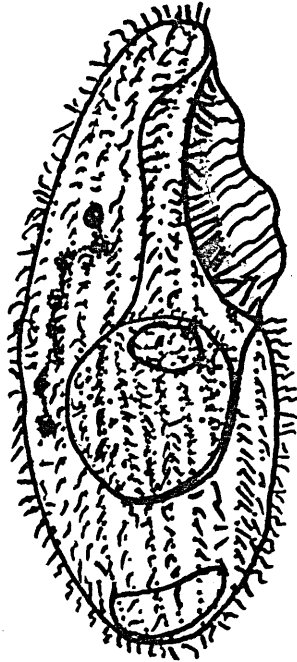
a.



b.



c.



d.

enclosed quickly within a food vesicle formed at the fundus of the cytopharyngeal pouch. Complete digestion of the enclosed prey requires 6-12 hours depending on the size of the prey.

Although prey were captured and ingested in every case observed, (N=10), the organisms did not always remain within the predator. In two cases, ingested B. americanum were able to turn around within the food vesicles and swim out of the mouth of the predator--apparently unharmed--after two minutes. Two other ingested B. americanum remained alive within the bodies of their respective predators for nearly two hours. One of these organisms eventually escaped when the predator was ripped open by a sharp piece of debris on the slide. The second organism was digested within the body of the second predator.

II. Changes in the Oral and Somatic Ciliature of Macrostomes as Compared to Microstomes.

Macrostomatous B. americanum previously treated with d-alpha-tocopheryl-succinate for 12 hours, were stained with either the protargol impregnation technique or the $\text{CrO}_3\text{-OsO}_4$ -glutaraldehyde method (see Materials & Methods III.) to reveal the somatic ciliary rows and the oral ciliature. The following data were obtained:

	<u>Controls</u>	<u>Giants</u>
Somatic ciliary row numbers	15 \pm 2	24 \pm 5
Length of Undulating Membrane	20 \pm 5 μm	45 \pm 5 μm
Width of Undulating Membrane	10 \pm 2 μm	25 \pm 5 μm
Length of Adoral Zone of Membranelles	50 \pm μm	80 μm

PLATE I:

Stages of Macrostomal Development of Blepharisma americanum Following Treatment of Microstomatous Organisms with 10^{-4} M d-alpha-tocopheryl succinate. (Study with the light microscope).

Fig. 1. A collection of untreated, microstomatous controls. Both the adoral zone of membranelles (AZM) and the undulating membrane (UM) are small and somewhat difficult to see at this stage.

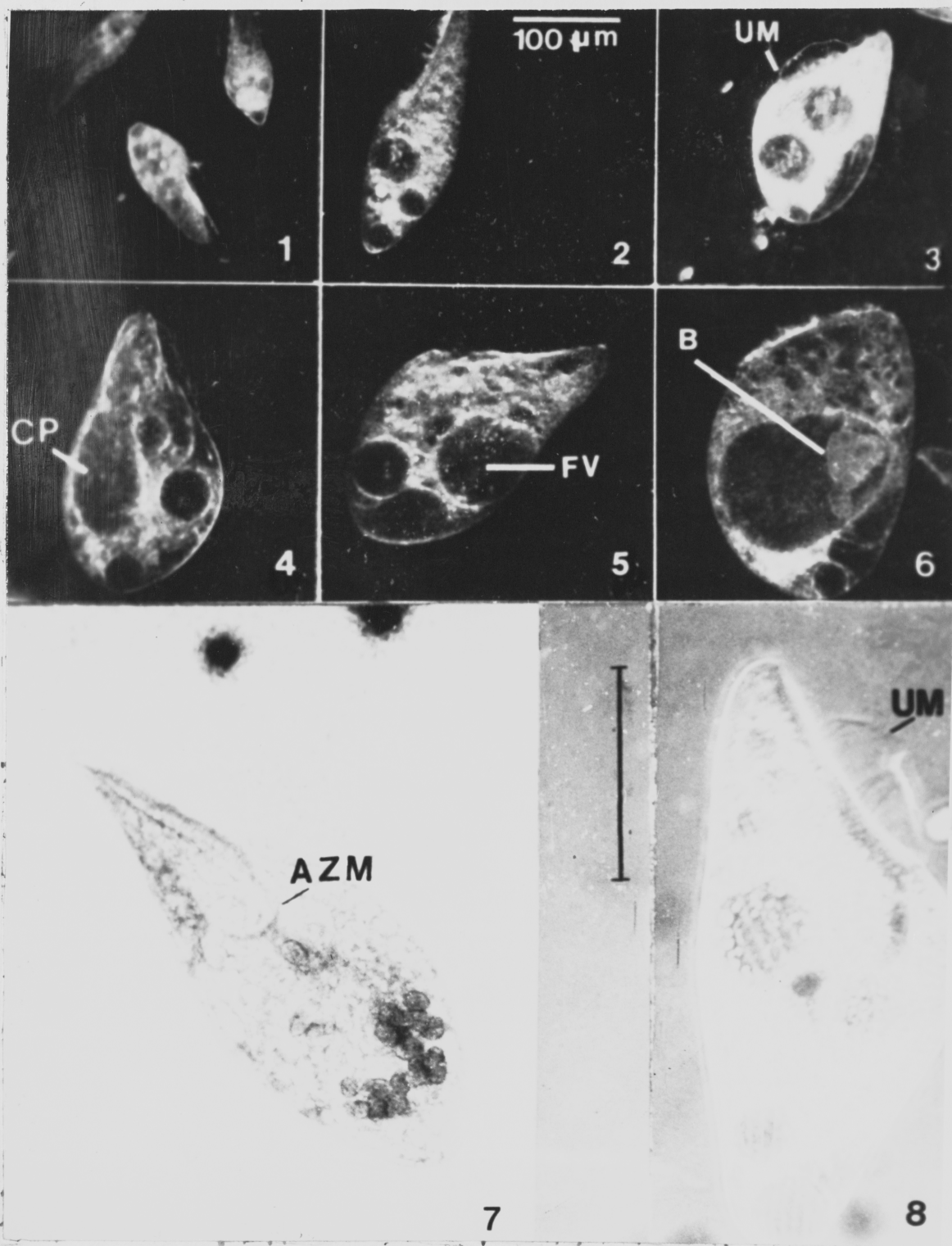
Fig. 2. An intermediate stage approximately 5-6 hours after the addition of the vitamin. The oral groove in this specimen is elongated.

Fig. 3. An early giant (9-10 hours following addition of the vitamin). The greatly enlarged undulating membrane (UM) and the cytopharyngeal pouch (CP) are easily seen.

Fig. 4. A giant form after 12 hours of exposure to vitamin E.

Figs. 5 & 6. A giant organism capable of ingesting smaller B. americanum (B). Food vesicles (FV) are prominent.

Fig. 7 & 8. A macrostome showing the characteristically enlarged undulating membrane, and the adoral zone of membranelles.



II. (Continued).

	<u>Control</u>	<u>Giant</u>
Width of the Adoral Zone of Membranelles	10 μ m	15 μ m

These measurements were made on 25 cells selected at random from both the controls and the tocopheryl-treated organisms. These results are comparable to earlier data compiled by Repak (1967), in his study of cortical changes of giant Blepharisma. In his study, however, the inducer was not defined.

III. Study of Macrostomal Development by Scanning Electron Microscopy.

The development of macrostomal morphology by B. americanum following treatment with d-alpha-tocopheryl-succinate, occurs by a series of changes of the oral ciliature and the buccal cavity. These changes can be most clearly followed by describing them in stages:

Stage I: Blepharisma americanum treated with vitamin E for 0-2 hours, are still typically microstomatous in appearance (Plate II, fig. 1). The adoral zone of membranelles (AZM) is composed of cilia 8-10 μ m in length. The undulating membrane (UM) is easily seen, but not highly conspicuous at this time.

Stage II: After 3-4 hours of exposure to the vitamin, transforming ciliates are either unchanged in overall length, or they may shorten from 150 μ m to 100 μ m. There is also a definite increase in the breadth of the anterior two-thirds

of the organism (Plate II, fig. 2). The oral groove decreases in length from approximately 70 μm to 50 μm . (Plate II, fig. 3 as compared to fig. 1.). In some organisms at this stage, the undulating membrane is somewhat shorter than in the controls, signaling the beginning of "oral replacement".

Stage III: During the 5th to the 7th hour of exposure of B. americanum to vitamin E, the oral groove begins to increase both in length (from 50 μm to 65 μm) and in breadth. The increase in breadth beginning at the posterior end of the oral groove is easily visible (Plate II, fig. 4). The length of the cilia comprising the oral apparatus is noticeably longer and there are more cilia in both the undulating membrane and the adoral zone of membranelles, as compared to controls. (Plate II, fig. 5).

Stages IV & V: Ciliates exposed to d-alpha-tocopheryl for 8-10 hours complete the development of a wide, deep oral groove and add still more cilia to the undulating membrane & to the adoral zone of membranelles (Plate II, fig. 6 & Plate III, fig. 2, arrows), along the latero-posterior margin. The undulating membrane is also considerably longer at this stage than in controls (Seen here after it has fallen into the oral groove during specimen preparation).

Reversion of the Macrostome to a Microstome:

When the vitamin is removed and B. americanum giants are resuspended in non-nutrient medium, reversion begins to occur after 24 hours. Plate III fig. 3 shows an organism beginning the first of 3 divisions that will ultimately produce microstomatous organisms. The first of these divisions is unequal;

PLATE II.

Stages of Macrostomal Development of Blepharisma americanum
as Revealed by Scanning Electron Microscopy (Scale bars
represent 10 μ m in all figures).

Fig. 1. A non-treated, microstomatous B. americanum showing the adoral zone of membranelles (AZM).

Fig. 2,3. A transforming organism 2.5-3.0 hours following the addition of vitamin E. The body of this organism and the oral apparatus are shortened as compared to controls. Also, the undulating membrane and the adoral zone of membranelles are shortened.

Fig. 4. An organism 4-5 hours following the addition of the vitamin. The oral groove becomes wider, beginning at the posterior end, and also increases in length.

Fig. 5. Both the adoral zone of membranelles (AZM) and the undulating membrane (UM) increase in width after 7-8 hours of exposure to the vitamin. Also, more cilia are added to each of these structures.

Fig. 6. A macrostome 8-10 hours following the addition of vitamin E has completed the development of a wide, deep oral groove suited to the capture of large prey.

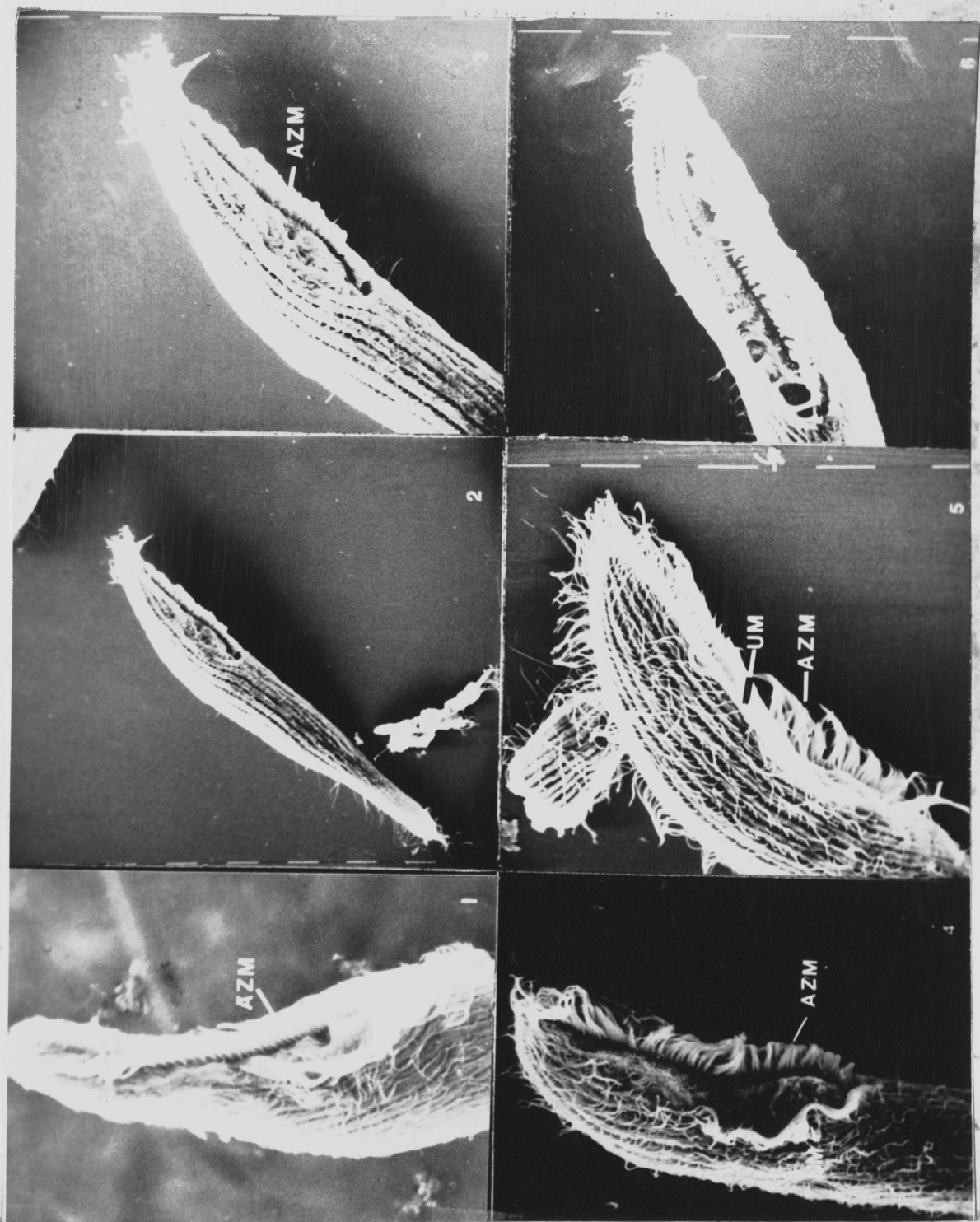


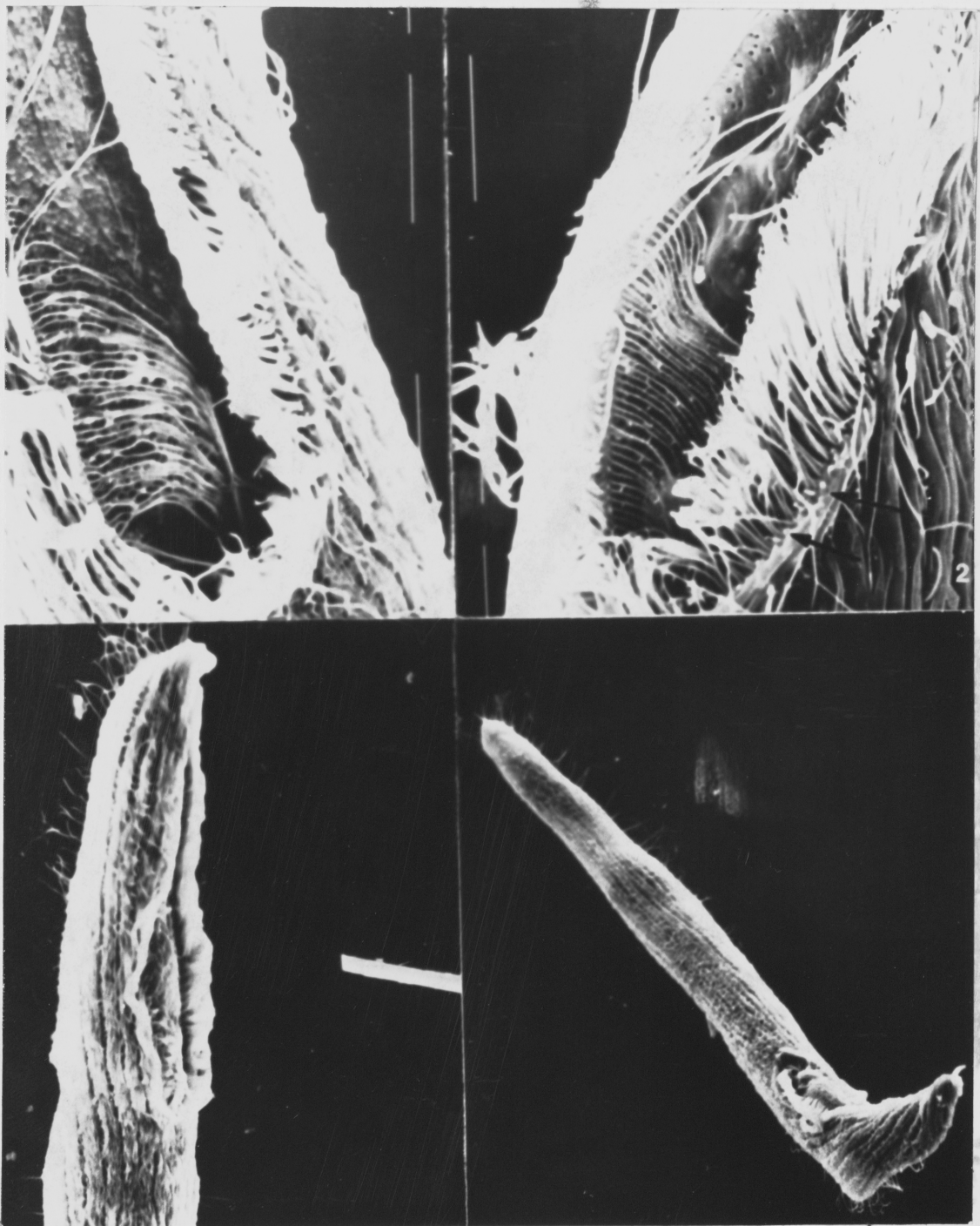
PLATE III:

Study of Macrostomal Production of B. americanum by
Scanning Electron Microscopy (continued). Scale bars = 10um.

Fig. 1. A macrostomatous B. americanum after 10-12 hours of exposure to the vitamin. The increased size and number of the oral cilia is apparent. An additional row of cilia is being added to the latero-posterior margin of the adoral zone of membranelles, seen in Fig. 2 (arrows).

Fig. 3. A macrostome that is beginning the first of 3 divisions that will result in the production of microstomatous organisms. The anterior cell is receiving the majority of the old ciliature. The posterior cell will reconstruct the oral ciliature from the remnant it receives.

Fig. 4. A representative L-shaped monster as it appears after 2.5 to 3.0 hours of exposure to the vitamin. Approximately 1% of the organisms in a given sample may assume this morphology.



the anterior daughter cell receives the majority of the oral ciliature, while the posterior daughter cell must reconstruct a complete oral apparatus from the remnant that it receives during division. The second division produces two microstomatous organisms of approximately equal size, although these are still somewhat larger than the controls. This is temporary, since the third division restores the proportions of a control organism.

L-Shaped Monsters:

A representative L-shaped monster is shown in Plate III., fig. 4. These forms appear together with organisms that have progressed to Stage II, but the L-shaped organisms do not form macrostomes at any time.

IV. Influence of Physical and Chemical Conditions on the Rate of Macrostomal Development By Blepharisma.

Treatment of microstomatous B. americanum with 10^{-4} M, 10^{-5} M, 10^{-6} M or 10^{-7} M d-alpha-tocopheryl-succinate resulted in the production of macrostomal morphology at different rates. Examination of Fig. 2a shows that concentrations of vitamin E from 10^{-4} M to 10^{-6} M, support the highest rates of development and 10^{-7} M is considerably less effective as an inducer. Untreated organisms did not transform into macrostomes during the experiment.

The rate of macrostomal development is also dependent on temperature, as shown in Fig. 2b. At 10°C , organisms exposed to 10^{-4} M d-alpha-tocopheryl-succinate transformed as far as Stage IV in 10-12 hours, although only 85% of the cells re-

sponded. At 20°C, 90% of the ciliates progressed as far as Stage IV in 10 hours, and at 30°C, 95-100% of the ciliates reached Stage IV in 7 hours. Control organisms did not become giants at any of the temperatures tested.

Previous studies of macrostomal development of Tetrahymena vorax V₂S have indicated that pH is an important factor in the microstomal to macrostomal transformation of this ciliate (Buhse, 1966 a). Therefore the effects of pH on the rate of macrostomal development of B. americanum were studied. The results of this experiment are depicted in Fig. 2c. These data indicate that the optimal range of pH for obtaining high yields of macrostomatous organisms is 5.0-5.5. Values above or below this range do not support the production of large numbers of macrostomatous organisms. Control organisms remained normal at each pH value throughout the experiment.

During the study of the effects of tocopheryl concentration on macrostomal production, it was noted that many stages occurred at the same time during the induction process. This observation lead to a study of the frequency distribution of these stages over time (Fig.3). Generally there was an assortment of stages until the last 2-3 hours of treatment with d-alpha-tocopheryl. From 10-12 hours, only Stages IV & V were seen.

Figure 2. The Effects of Physical and Chemical Conditions on the rate of macrostomal development by Blepharisma americanum treated with d-alpha-tocopheryl-succinate. a. The effects of the concentration of tocopheryl used as an inducer (values are percentage of macrostomes formed vs. time. Concentration units are moles/liter. b. The effects of temperature on the rate of transformation from microstome to macrostome. c. The influence of pH on the rate of macrostomal production.

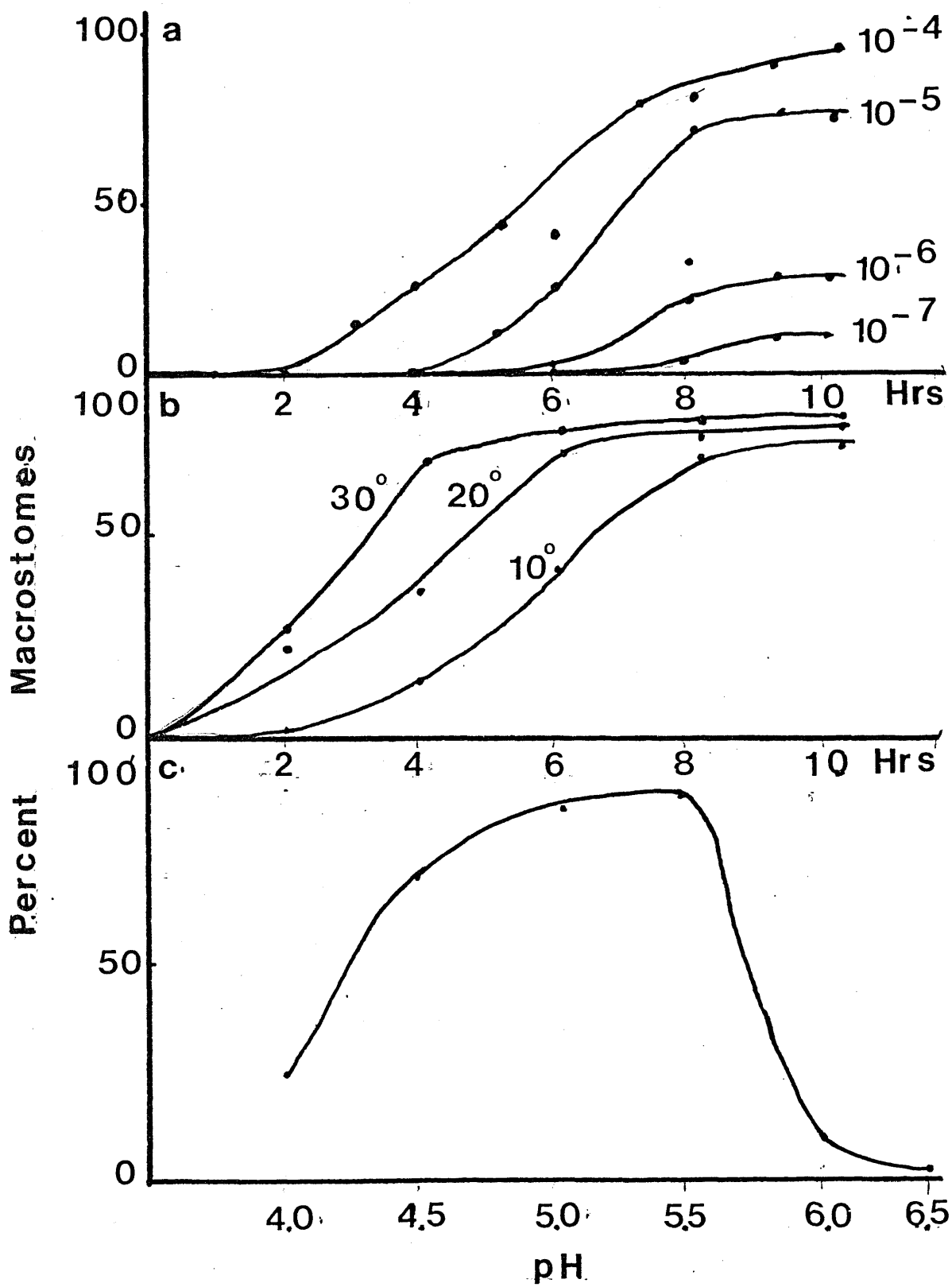
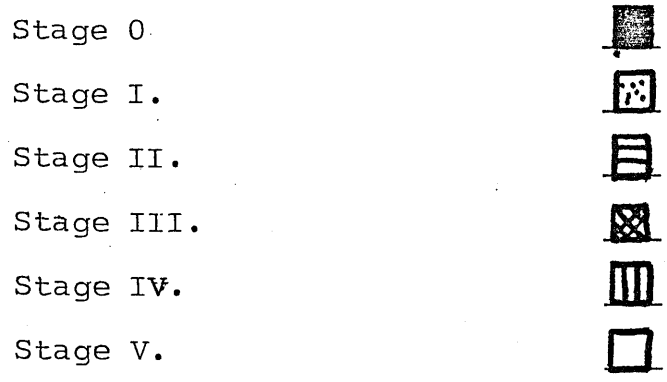
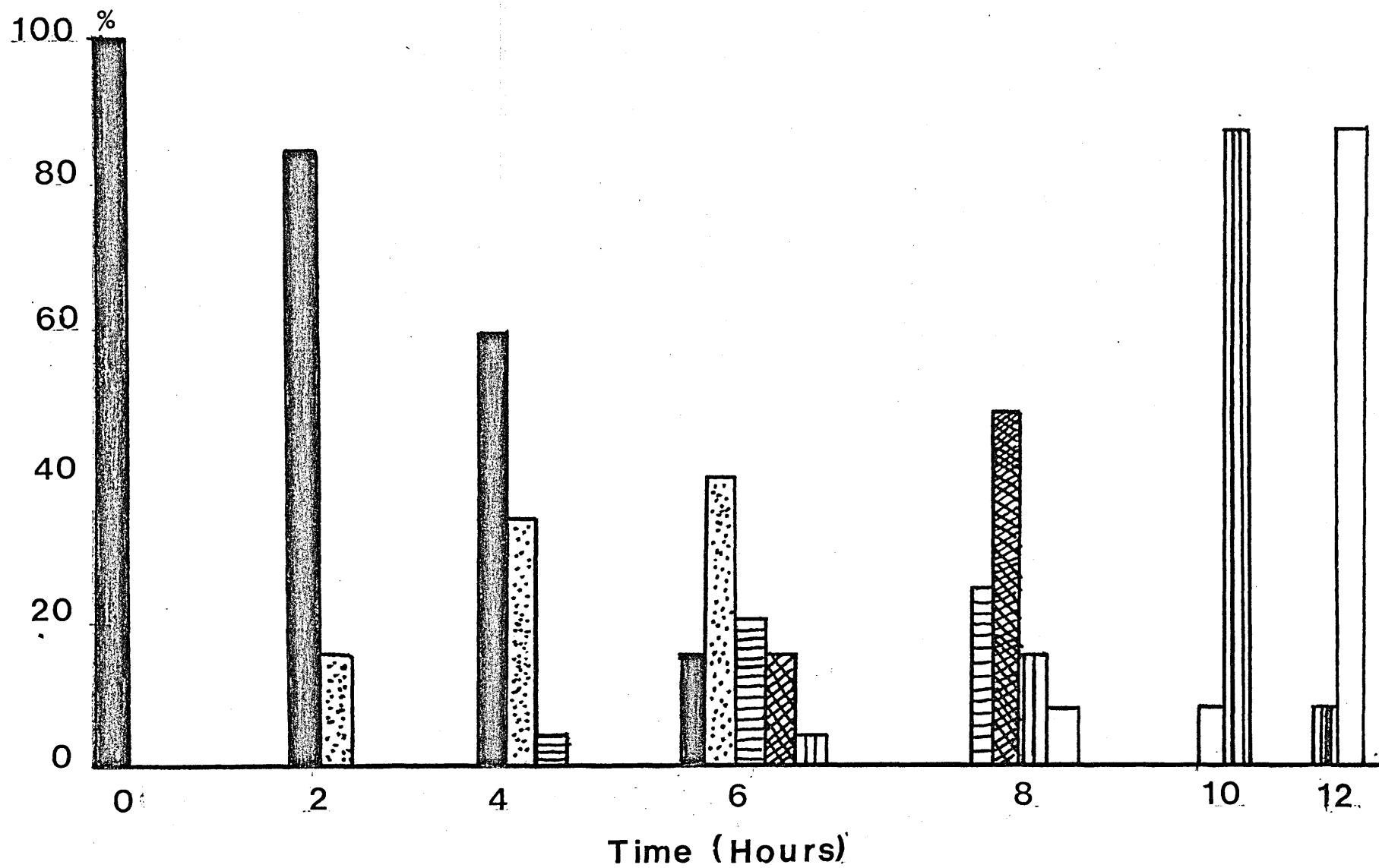


Figure 3. Frequency Distribution of Stages that occur during macrostomal development by Blepharisma americanum following treatment of microstomatous ciliates (Stage 0) with 10^{-4} M d-alpha-tocopheryl-succinate.





V. The Effects of Starvation on Macrostomal Development.

The microstomatous B. americanum used as controls for the experiment remained in that form throughout the experiment and fed on bacteria.

In contrast, ciliates previously fed with bacteria and then treated with 10^{-4} M d-alpha-tocopheryl-succinate, transformed into macrostomes within 10 hours. Some cannibalism was noted in this group.

Ciliates that had been starved, but not treated with vitamin E, became very thin and fragile. Many of these ciliates cytolized during the experiment. If these organisms moved at all, they were sluggish.

In the last group, organisms were starved for 6, 12 or 24 hours prior to the addition of d-alpha-tocopheryl-succinate. Those starved for 6 hours prior to treatment, progressed as far as Stage III. Only 50% of the organisms responded. Organisms starved for 12 hours responded to the vitamin E by enlarging somewhat, but they did not progress toward giantism. Ciliates starved for 24 hours prior to treatment with the vitamin did not respond. They were sluggish and did not feed extensively, but they did not cytolize during the experiment.

VI. Studies of Ultrastructural Changes of Macrostomes.

The ultrastructural features of non-treated controls were compared with those of Stage IV-V macrostomatous B. americanum, to see if changes related to the development of macrostomal morphology had occurred in organisms treated with d-alpha-tocopheryl-succinate.

Ciliature and Microtubules: These structures did not demonstrate differences that could be attributed solely to d-alpha-tocopheryl succinate. However, a more detailed study is needed to gain further information, since scanning electron microscopy does indicate that there are changes in the ciliature.

Mitochondria: The tubular cristae typical of many mitochondria from protozoa were noted in sections from B. americanum. There were a variety of shapes and sizes in both the control and treated samples, so no significant differences could be attributed to vitamin E treatment as compared to the controls.

Rough Endoplasmic Reticulum: There were numerous sections wherein the rough endoplasmic reticulum of macrostomes had more ribosomes per unit area than the untreated controls. The ribosomes were of the same diameter as particles found in the nucleus.

Macronucleus: Macronuclei of controls had few, if any nucleoli. When present, these organelles measured 0.2-0.3 um in diameter. They were granular in overall appearance (Plate IV, fig. 1 & 5).

In contrast, the macronuclei of macrostomatous organisms contained a significantly greater number of nucleoli than the untreated controls [0-4, \bar{x} = 3 for the controls and 4-17, \bar{x} = 11 for the treated cells]. The nucleoli from giants were also significantly larger than those found in the macronuclei of the controls, [0.4 to 2.0 μ m \bar{x} = 0.67]. Many of these nucleoli each showed distinct clusters of granules around the periphery. In close association with these clusters; chains of particles strongly resembling ribosomes are seen, [Plate IV, fig. 4 and 6,7].

In an experiment performed to clarify the nature of the particles, a sample of tocopheryl-treated macrostomes was exposed to puromycin (100 mcg/ml./45 min.), an inhibitor of peptide chain elongation. The inhibitor was removed and then both this sample and an untreated control sample were prepared for transmission electron microscopy. The results of this experiment are shown in Plate IV, fig. 3. No chains of particles such as those shown in Plate IV, fig. 4, were detected. Further, no more than one structure clearly identifiable as a nucleolus was seen in the puromycin-treated section. The macronuclei also displayed less intensely stained chromatin, suggesting that a protein(s) normally associated with the DNA is absent or greatly reduced in total amount

There are somewhat greater numbers of nuclear pores in the macronuclei of organisms treated with tocopheryl than in the macronuclei of controls. This is also true for the micro-nucleus.

PLATE IV:

Changes of Macronuclear and Nucleolar Morphology of Blepharisma americanum Following Exposure to Vitamin E, as Revealed by Transmission Electron Microscopy.

Fig. 1. A section through a control showing the macronucleus (M) and the micronucleus (m). Nucleoli are inconspicuous.

Fig. 2. A section through a macronuclear node of an organism after 6 hours of exposure to d-alpha-tocopheryl. Nucleoli (N) are enlarged as compared to the control and there are also more nucleoli present. A micronucleus is also shown (m).

Fig. 3. A macronuclear node of a macrostomatous ciliate after it has been exposed to puromycin (100 mcg/ml/45 min.). There is only one structure that resembles a nucleolus. The chromatin is less intensely stained than in either Fig. 1 or 2.

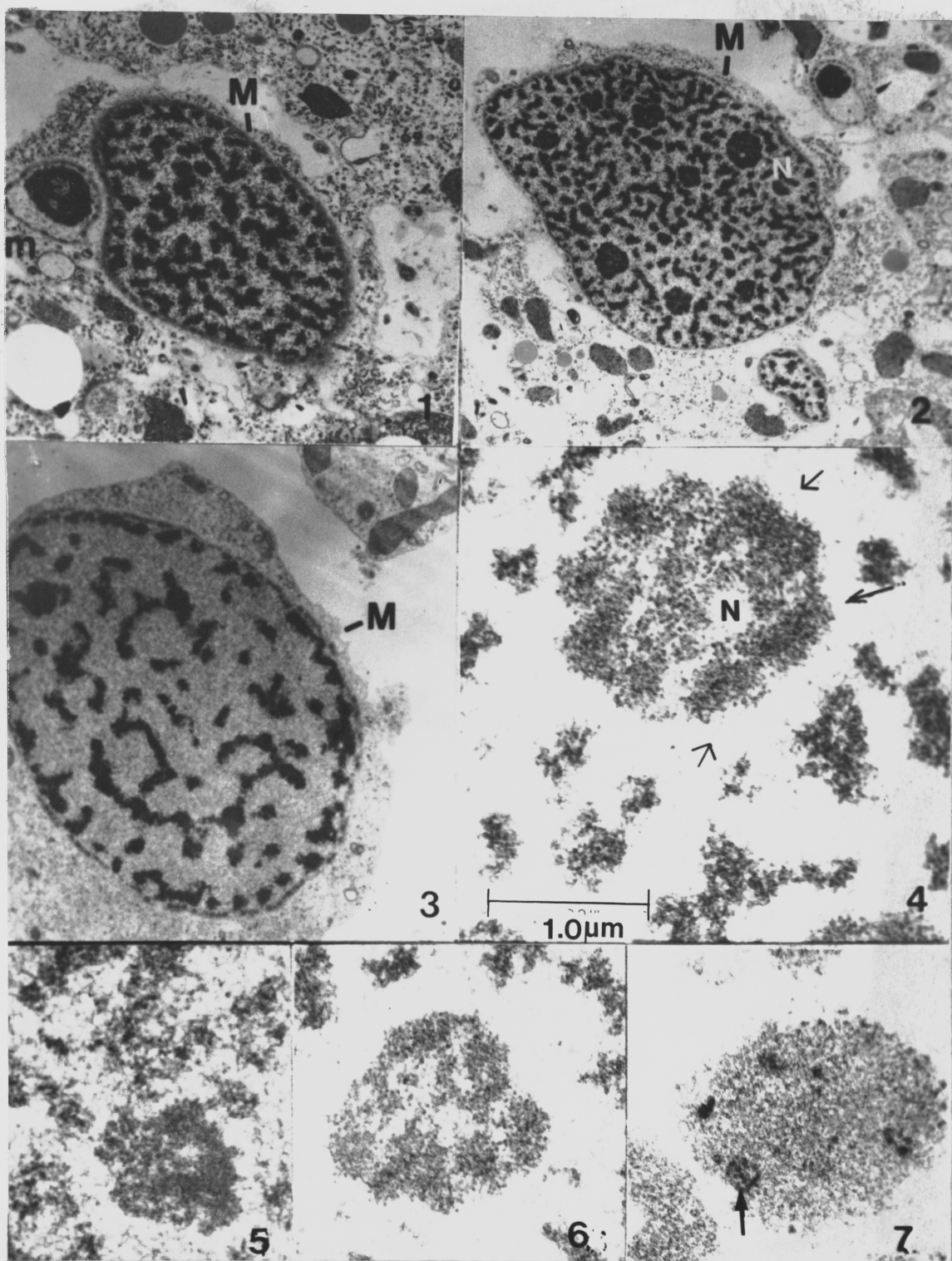
Fig. 4. A nucleolus showing chains of ribosomes (arrows)

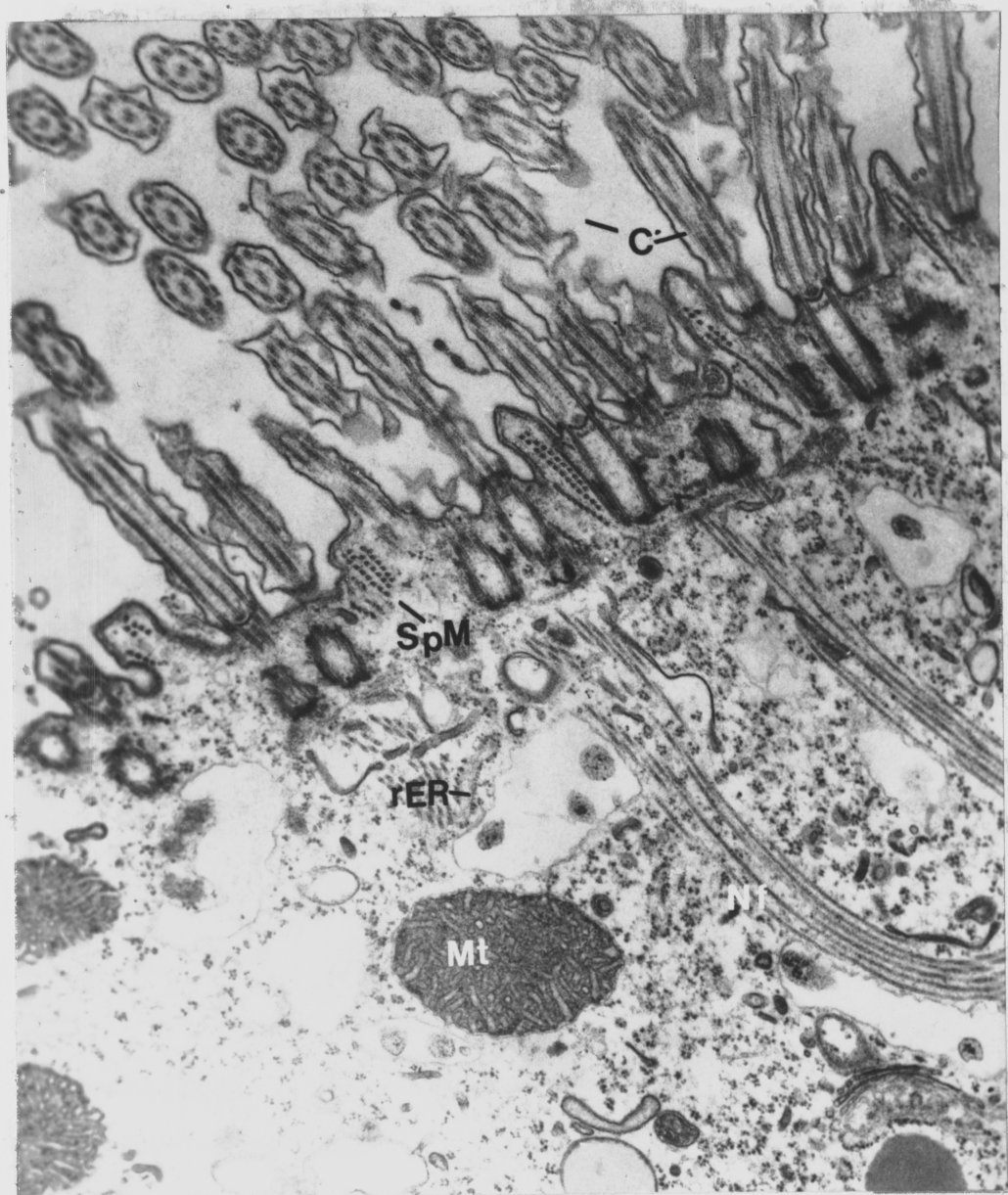
Fig. 5. A nucleolus from a control. Fig. 6. and Fig. 7. Nucleoli from macrostomes, showing nucleolus associated chromatin (arrow).

PLATE V:

A Survey of Macrostomal Ultrastructure By Transmission Electron Microscopy.

This figure shows cilia (C) of the adoral zone of membranelles as well as subpellicular microtubules (SpM), mitochondria (Mt) and the nemadesmal fibers associated with cilia, (Nf). The rough endoplasmic reticulum (rER) is also present.





VII. An Initial Study of the Dependence of Macrostomal Development and Maintenance on Protein Synthesis.

When Blepharisma americanum was exposed to puromycin (100 mcg/ml for 45 minutes) during treatment with d-alpha-tocopheryl-succinate (10^{-4} M), macrostomal development occurred after 14-16 hours. Eighty percent of the organisms progressed as far as Stage IV, while the remaining ciliates did not change. Control organisms, treated solely with vitamin E, became giants within 10 hours. Ninety percent of the controls responded and the rest were microstomatous.

When puromycin was added to a sample of organisms 6 hours after the beginning of treatment with vitamin E, approximately 60% of the ciliates enlarged, but these did not become giants. These ciliates did not form the characteristic cytopharyngeal pouch and their oral ciliature was also somewhat shorter than that formed by the macrostomatous giants in the absence of puromycin.

In a separate experiment, puromycin was added to a sample of completed macrostomes. Within 20 minutes, the cytopharyngeal pouch collapsed in nearly every organism. When the inhibitor was removed, the pouch was reconstructed in 85% of the cells within 90 minutes.

In contrast to the inhibitory effects of puromycin, the addition of cycloheximide (5mcg/ml) prevented the formation of macrostomes in the presence of tocopheryl-succinate. This effect was reversible, although only 50% of the ciliates completed macrostomal development. Also, the oral ciliature was shorter than that formed by macrostomes in the absence

of the drug. When cycloheximide was added to a sample of completed macrostomes, the cytopharyngeal pouch collapsed within 20 minutes. If the drug was removed, the cytopharyngeal pouch was reconstructed in 50% of the cells in 90 minutes.

VIII. Study of the Effects of Colchicine.

Addition of colchicine (10^{-6} M final concentration) resulted in the collapse of the cytopharyngeal pouch of macrostomatous organisms within 15 minutes. When the inhibitor was removed, the ciliates reconstructed the pouch after 2-3 hours of additional exposure to 10^{-4} M d-alpha-tocopheryl. Ninety five percent of the ciliates were affected by the inhibitor and of these, 85% were able to reconstruct the pouch after the inhibitor was removed.

IX. Induction of Macrostomal Development by Butylated Hydroxytoluene (BHT).

In order to ascertain whether or not a non-vitamin antioxidant might be capable of inducing macrostomal development of B. americanum, a set of experiments using BHT were conducted.

When samples of ciliates were exposed to a series of concentrations of BHT, 10^{-4} M (pH = 5.0-5.5, Temperature = 25°C) induced the largest percentage of transformation in 12 hours (95%). Lower concentrations, (10^{-5} M, 10^{-6} M & 10^{-7} M), resulted in 40%, 25% & 5% transformation, respectively, over the same span of time.

A study of the effects of temperature showed that at each of the temperature tested, (10°C, 20°C & 30°C), B. americanum transformed into the macrostomal type. At 10°C, this occurred after 16 hours; at 20°C, 12 hours and at 30°C, 9 hours. In each case, 90% of the cells became enlarged while the rest were unchanged.

The untreated controls used for each of the above studies did not change during the experiments.

A significant difference between the effects of this antioxidant and the effects of d-alpha-tocopheryl-succinate is that macrostomal maintenance in the presence of BHT was transitory. After 3-4 hours, macrostomes reverted to the microstomatous form. This was possible because none of the organisms attained the full extent of giantism that is possible following treatment with d-alpha-tocopheryl-succinate.

X. An Initial Study of the Prevention of Damage to Cell Membranes From Ultraviolet Radiation, By Vitamin E.

When microstomatous B. americanum were exposed to ultraviolet radiation (254 nm), the organisms cytolized within 10 seconds. [Total dosage=36.67 Joules/m². Exposure time 5-10 seconds]. This was repeated three times using 50 cells per sample. The results were always the same none of the ciliates survived. In several organisms, pigment capsules were shed but this did not protect them.

When a second set of organisms were exposed to 10⁻⁵M d-alpha-tocopheryl succinate for 6 hours and subsequently washed and resuspended in Chalkley's solution, the results of irradiation with the same dosage of ultraviolet radiation was quite different. For 95% of the ciliates in this set of 3 samples, the irradiation did not result in cytolysis. The ciliates became somewhat longer and thinner than prior to the experiment, but no other changes were noted. These cells were used to initiate clonal cultures. The interdivision period was found to be approximately 30 hours for these organisms as compared to 20 hours for non-treated controls. Since this was also true of organisms that received only treatment with vitamin E, the longer period of time between divisions was not due to ultraviolet irradiation.

IV.

DISCUSSION AND CONCLUSIONS

Protozoa are excellent tools for the study of cellular processes. In particular, their relatively large size, and the occurrence of easily visible changes in the patterns of their ciliature, makes ciliates an ideal choice for many studies of cellular morphogenesis.

An example of the utility of ciliated protozoa for studies of morphogenic transformations of cells, is provided by the study of macrostomal development of Blepharisma americanum. The recently devised technique of exposing microstomatous B. americanum to d-alpha-tocopheryl-succinate (vitamin E), in order to produce the macrostomatous form of this organism (Lennartz & Bovee, 1980), provides a novel approach to the critical study of both the structural and the biochemical events that precede the development of macrostomes, as well as the basis for the maintenance of the macrostomal phenotype. Since B. americanum responds to treatment with d-alpha-tocopheryl-succinate by undergoing a cellular transformation, study of the phenomenon of macrostomal development is also a useful approach to the understanding of the role of this vitamin in cellular metabolism.

Macrostomal Development as an Alternative to Cell Division.

The transformation of tocopheryl-treated B. americanum from the microstomatous form to the macrostomatous form, can be viewed as the selection of an alternative developmental pathway. Observations from this study of macrostomal development of B. americanum treated with d-alpha-tocopheryl, as well as previous studies of several members of the genus

Blepharisma (Giese, 1938; Hirshfield, et al, 1963; Repak, 1967; Repak et al, 1978 & Steinberg, 1959), indicate that cellular division is inhibited during macrostomal development. Studies of macrostomal development in Tetrahymena vorax, V₂S (Buhse, 1967), T. paravorax (Metenier, 1978), & T. patula (Stone, 1963), conclude that cellular division is inhibited during macrostomal development of these ciliates as well. Another factor involved in the process of switching from a "division pathway" to macrostomal development is the occurrence of a "competence period" during the cell cycle, i.e., a period of time (approximately one hour long) wherein an organism can be induced to follow the pathway toward macrostomal development, as opposed to the pathway leading to cellular division. At times prior to the competence period, or subsequent to this time, macrostomal development cannot be induced. This "temporal restriction" of morphogenic pathways has been documented previously in T. vorax V₂S (Metenier, 1978). The existence of a competence period for the transformation of Blepharisma americanum from the microstome to the macrostome form is indicated by two observations from the present studies. First, under otherwise standardized conditions, induction of macrostomal development by treatment of microstomes with d-alpha-tocopheryl succinate is not always successful. Further, the transformation process is not necessarily synchronous within a given sample--many stages may occur simultaneously at any given time after the addition of the vitamin. (see Fig. 3). In T. vorax, the relationship between the com-

petence period and the cell cycle has been determined; the competence period occurs during early G₂ and lasts for approximately one hour (Metenier, 1978). This relationship has not yet been determined for B. americanum.

Morphogenic Events of Macrostomal Development.

Macrostomal development by B. americanum treated with d-alpha-tocopheryl succinate occurs via a marked enlargement of the oral cavity and of the oral ciliary organelles.

The component cilia of the undulating membrane and of the adoral zone of membranelles increase in length during macrostomal development. This may be due to the incorporation of newly synthesized structural proteins of cilia, such as tubulins, the utilization of pre-existing "pools" of such proteins, or to a combination of both mechanisms. When B. americanum was starved prior to exposure to d-alpha-tocopheryl, transformation did not occur. If either puromycin or cycloheximide was added during the transformation, protein synthesis was inhibited, and the macrostomes formed had shorter oral cilia. These results suggest that oral ciliature increases in length due to the utilization of precursor pools and de novo synthesis of ciliary proteins.

In addition to increasing in length, oral cilia increase in number. The adoral zone of membranelles acquires an additional row of cilia by the addition of a row of kinetosomes, beginning at the posterior margin of the adoral zone of membranelles and proceeding toward the anterior end of the oral apparatus. This observation stands in contrast to an earlier

view that kinetosomes are added at the anterior end of the adoral zone of membranelles of developing macrostomes (Repak, 1967). The means whereby the undulating membrane acquires additional cilia is not known.

The production of an enlarged oral ciliature is accompanied by the formation of a greatly enlarged oral groove and buccal cavity. Observations of transforming B. americanum by light microscopy show that a "cytopharyngeal pouch" is formed approximately 6 hours after the addition of $10^{-4}M$ d-alpha-tocopheryl-succinate. This structure is formed by the coalescence of a large, centrally positioned vesicle with the posterior portion of the cytopharynx. In this respect, macrostomal development of B. americanum is similar to macrostomal production of Tetrahymena vorax (Kidder, 1940; Buhse, 1966a; Buhse et al, 1978). The expansion of the oral groove, as observed with the scanning electron microscope, begins at the posterior end of the oral apparatus and continues toward the anterior end of the organism. This expansion may be due to the rearrangement of subpellicular microtubules as the cytopharyngeal pouch begins to form. This contention is supported by the observation that treatment of nearly completed macrostomes with colchicine, an inhibitor of microtubular synthesis, caused collapse of the cytopharyngeal pouch, and of the expanded oral groove, within 10-15 minutes. Changes in the subpellicular fibril system may also explain the production of the observed L-shaped monsters.

The Role of Protein Synthesis in the Formation and Maintenance of Macrostomal Morphology by *Blepharisma americanum*.

The concept that protein synthesis is required for the production and maintenance of the macrostomatous morphology of polymorphic ciliates developed from studies of *Tetrahymena vorax*, wherein specific inhibitors of protein synthesis were used (Buhse & Nicolette, 1969; Buhse et al, 1978; Nicolette et al, 1971). The need for continuous protein synthesis during tocopheryl-induced macrostomal development of *B. americanum* is shown by the results of the addition of cycloheximide to samples of transforming cells. When this inhibitor of peptide chain elongation (Lewin, 1974) was added prior to 6 hours following the addition of vitamin E, ciliates were not able to produce macrostomes. When the inhibitor was removed, the process of macrostomal formation resumed after a delay of about 3 hours.

When macrostomatous *B. americanum* were treated with either puromycin (100 mcg/ml) or cycloheximide (5 mcg/ml), the cytopharyngeal pouch collapsed in nearly every cell. The effects of these drugs could be reversed by washing them off and resuspending the ciliates in 10^{-4} M d-alpha-tocopheryl succinate. Similar recovery has been observed for *Tetrahymena vorax* V₂S with the uncharacterized substance, "stomatin", serving as the inducer (Buhse, et al, 1978). These studies indicate that continuous synthesis of a protein(s) is required for the maintenance of the macrostomal morphology of *B. americanum*.

An additional type of evidence emphasizing the importance of protein synthesis for the development and maintenance of macrostomal morphology by B. americanum, is derived from a study of the ultrastructure of the macronuclear nodes of this ciliate.

Electron-micrographs of macronuclear nodes from macrostomatous organisms show a significant increase in both the size and the number of nucleoli, as compared to microstomatous control ciliates. Since nucleoli are known to be centers of ribosome production, and ribosomes are necessary for the synthesis of proteins, it is reasonable to conclude that d-alpha-tocopheryl acts as a modulator of transcription. In the present case, those portions of the genome that are responsible for the synthesis of nucleolar and ribosomal components have been activated to meet the need for increased biosynthetic activities required of the giant ciliate. This amplification of transcription has previously been noted in such organisms as amphibians (Gall, 1968) and insects (Gall, et al, 1969).

Further support for the contention that increased numbers of ribosomes are being synthesized by macrostomes as compared to microstomes, is shown by electron-micrographs of individual nucleoli [see Plate IV, fig. 4, 6, 7]. Most of the nucleoli observed in sections of tocopheryl-treated B. americanum show ribosome-like and polysome-like particles (125 angstroms in diameter). These particles are similar in size to the ribosomes that are associated with the rough endo-

plasmic reticulum found in the cytoplasm. Another observation supporting the probable ribosomal nature of these particles is that the density of particles associated with the endoplasmic reticulum of giant B. americanum is greater than the density of such particles seen in sections from untreated controls. This suggests that ribosomes are being transported from the macronuclear nodes to the cytoplasm in increased numbers to provide greater amounts of protein for the giant ciliate. Since these particles are not visible from sections of macrostomes previously treated with puromycin, an inhibitor of polypeptide chain elongation (Lewin, 1974), their ribosomal nature is confirmed. Nucleoli of untreated B. americanum do not show amplification in either number or size. Also, the chains of particles are either absent or infrequent.

Nucleoli from other protozoa, actively engaged in biosynthesis, also show particles and chains of particles that are very similar to those observed in the present study (Frenkel, 1980; Kaneda, 1961; Kluss, 1962; Krawczynska, 1980 & Seshachar, 1964). In addition, chains of ribosomes have also been observed in higher eucaryotes (see Lane, 1967 for her study of the axolotl oocyte).

A Model for the Mechanism of Action of Vitamin E as an Inducer of Macrostomal Development By Blepharisma americanum.

The results of the present studies can be used to formulate a plausible model for the mechanisms of action of d-alpha-tocopheryl as an inducer of macrostomal development.

The first part of the model suggests that the vitamin enters the organisms through both the cell membrane and the cytostome. Some of the tocopheryl molecules become associated with the polyunsaturated fatty acid components of the cell membrane (e.g. arachidonic acid, see Machlin, 1980) & act as "radical scavengers". Thus, during the oxidative reactions that occur during the exposure of B. americanum to ultraviolet radiation, the organisms do not cytolyze (see Results, Section X). Studies using radioactively labeled tocopheryl could be done to further substantiate this portion of the model. The autoradiographic methods are currently available.

While some of the vitamin remains associated with the cell membrane, the rest of the tocopheryl might become bound to a specific "binding protein" within the cytosol. This protein would probably be globular and similar to that found by Jaharan, et al (1973) in their study of the cytosol fraction of rat liver cells. In this form, the vitamin might be able to enter the macronucleus and there become associated with the DNA. This could be established by autoradiographic tracking of the tocopheryl molecules using a "pulse-chase" procedure similar to that used for studies of the binding of

hormones to the DNA of other eucaryotes. Also, a similar study using labeled tocopheryl with cells from the liver (rat), has shown that tocopheryl does associate with DNA (Hauswirth & Nair, 1972).

Once inside the macronucleus, the tocopheryl could initiate changes in the transcription of the genome and thereby alter the synthetic pathways of the organisms toward giant formation. One approach to test this hypothesis would entail searching for new proteins synthesized in the macrostome that were not present prior to the addition of tocopheryl. A technique known as two dimensional gel electrophoresis could be used to find such proteins. This method has been successfully used for the isolation of proteins from the oral apparatus of microstomatous Tetrahymena pyriformis (Gavin, 1980) and could be modified for use with other ciliates as well. The approach mentioned above could be combined with the technique of using inhibitors of RNA synthesis (i.e. actinomycin D) to determine whether the effects of tocopheryl are directed toward transcriptional control or toward some other segment of the protein synthesis process. By treating cells with tocopheryl and then sampling at predetermined times, the chronological relationships between RNA synthesis, protein synthesis and macrostome production could be determined.

If this model, or a modified form of the model, can be verified, such information would permit us to more fully understand how organisms make use of external "cues" in order to evaluate the condition of their environment and, when necessary, respond to those signals in order to survive.

v.

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APPENDIX A
FORMULARY OF SOLUTIONS

CHALKLEY'S SOLUTION

This salt solution was used throughout these studies for a variety of purposes; culture media, washing fluid and as a component for antioxidant solutions. It is compounded with the following substances:

NaCl	0.1 grams
KCl	0.004 grams
CaCl ₂	0.006 grams
Double Distilled H ₂ O	1.000 liter

CrO₃--OsO₄--GLUTARALDEHYDE FIXATIVE

The following fixative solution was developed for use with Blepharisma americanum. This fixative acts rapidly, stains oral ciliature and basal bodies and may be used for either light or scanning electron microscopy. The constituents are as follows:

OsO ₄ (4% aqueous)	1 part
CrO ₃ (0.5-1.0% aqueous)	1 part
Glutaraldehyde (2.5% in 0.1 M Na-cacodylate buffer @ pH=7.4)	2 parts

The fixative is prepared immediately before use and may be employed over a temperature range of 10-25°C for 15-60 minutes (shorter exposures are usually preferred).

APPENDIX B
EXPERIMENTAL DATA
TABLES

Table II.

Concentration Dependence of Tocopheryl-Succinate
Induced Macrostomal Development of Blephar-
isma americanum (Percent Macrostomes)

10^{-4} M d-alpha-tocopheryl succinate

Time (Hrs)	Trial #1	Trial #2	Trial #3	Mean
0	0	0	0	0
1	0	0	0	0
2	0	2	4	2.0
3	18	14	14	15.3
4	24	24	26	24.7
5	40	46	42	43.0
6	50	50	46	48.7
7	78	72	72	75.0
8	84	84	72	80.0
9	96	90	90	92.0
10	94	98	96	96.0
11	94	98	96	96.0
12	94	98	96	96.0

10^{-5} M d-alpha-tocopheryl succinate

0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	2	2	0	1.3
4	10	8	8	8.7
5	10	10	8	8.7
6	14	14	10	12.7
7	24	22	18	21.3
8	30	40	40	36.7
9	50	50	40	46.7
10	70	64	78	70.7
11	70	64	78	70.7
12	70	64	78	70.7

10^{-6} M d-alpha-tocopheryl succinate

0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	2	0	0	0.67
6	0	0	0	0.67
7	4	10	4	6.0
8	20	24	10	18.0
9	24	24	24	24.0
10	24	24	24	24.0
11	24	26	24	24.7
12	24	28	26	26.0

Table II (Continued)
 10^{-7} M d-alpha-tocopheryl succinate

Time (Hrs.)	Trial #1	Trial #2	Trial #3	Mean
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	8	4	8	6.67
10	8	8	8	8.0
11	8	8	8	8.0
12	8	8	8	8.0

Non-treated ciliates did not change toward macrostomes at any time during the experiment.

Table III:
Influence of Temperature on Macrostomal
Development of Blepharisma americanum
(Expressed as Percent Macrostomes Formed)

Temperature=10°C				
Time (Hrs.)	Trial #1	Trial #2	Trial #3	Mean %
0	0	0	0	0.
1	0	0	0	0
2	0	2	0	0.67
3	1	2	2	1.6
4	15	16	16	15.6
5	30	32	28	30.0
6	45	42	43	43.3
7	62	60	64	62.0
8	85	80	88	84.0
9	85	85	90	86.7
10	85	85	90	86.7
11	85	85	90	86.7
12	88	85	90	87.7

Temperature=20°C				
0	0	0	2	0.67
1	5	8	7	6.67
2	12	15	18	15.0
3	20	28	26	25.0
4	40	36	38	37.7
5	60	52	60	57.0
6	66	66	70	67.3
7	85	80	78	81.0
8	85	85	80	83.0
9	80	90	86	85.0
10	80	90	90	86.7
11	90	90	90	86.7
12	90	90	90	86.7

Temperature=30°C				
0	0	2	4	2.0
1	5	10	20	11.6
2	20	20	18	19.3
3	40	44	44	42.7
4	65	70	78	71.0
5	80	80	82	80.6
6	85	90	82	85.6
7	90	90	88	90.0
8	88	90	99	92.3
9	99	90	88	92.3
10	99	95	95	95.6
11	99	95	95	95.6
12	99	95	95	95.6

Table IV:
Influence of pH on Macrostomal Induction
of Blepharisma americanum by
d-alpha-tocopheryl-succinate

pH	Percent Macrostomes Formed			Mean %
4.0	20	25	30	25.0
4.5	77	75	70	74.0
5.0	100	94	98	97.3
5.5	95	95	97	95.0
6.0	10	18	20	16.0
6.5	5	5	5	5.0

All experiments were performed using 10^{-4} M d-alpha-tocopheryl-succinate at 25°C. 50 cells per sample were used.

Time for each experiment was 10.0 hours.

Controls consisting of either 1% ethanol, 10^{-4} M succinate, or a combination of each substance (1% with respect to the ethanol and 10^{-4} M with respect to succinate), were also tested at each pH. None of these controls showed changes toward macrostome production.